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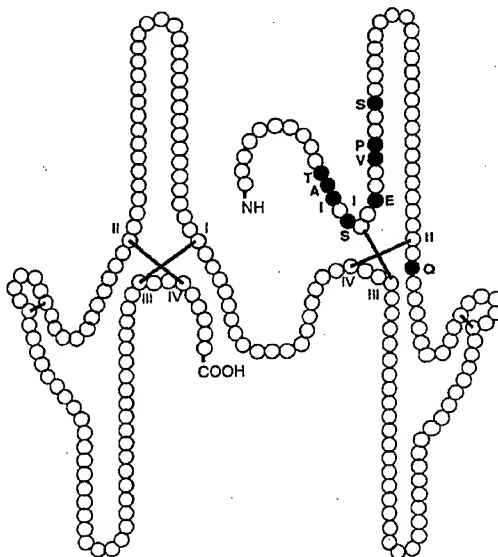
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(54) Title: IMMUNOGENIC REGION RECOGNIZED BY CIRCULATING AUTOANTIBODIES FROM GOODPASTURE PATIENTS

(57) Abstract

The invention refers to an immunogenic region comprising a part of the non-collagenous domain of the $\alpha 3$ chain of type IV collagen having intact cysteine residues, said part being recognized by circulating autoantibodies from Goodpasture patients. This part comprises at least five amino acid residues which are substantially non-adjacent in the amino acid sequence, it is located in the amino terminal end of said $\alpha 3$ chain, and it is exposed to said autoantibodies. The invention also refers to methods for the determination in body fluids of circulating autoantibodies from Goodpasture patients.



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**Immunogenic region recognized by circulating autoantibodies
from Goodpasture patients**

The invention refers to the immunogenic region of the
5 non-collagenous domain of the $\alpha 3$ chain of type IV collagen.
More specifically, the invention refers a part of the non-
collagenous domain of the $\alpha 3$ chain of type IV collagen
having intact cysteine residues, said part being recognized
by circulating autoantibodies from Goodpasture patients.
10 The invention also refers to methods for the *in vitro*
determination in body fluids of circulating autoantibodies
from Goodpasture patients.

Goodpasture's syndrome is a prototype autoimmune
disease characterized by the formation of pathogenic auto-
15 antibodies directed against the basement membrane collagen
type IV. The formation of autoantibody-antigen complex in
the glomerular basement membrane causes a rapidly pro-
gressive glomerulonephritis often accompanied by life-
threatening pulmonary hemorrhage.

20 Since Goodpasture's syndrome is a classic autoimmune
disease it fulfills all criteria in analogy to Koch's
postulate for infectious diseases. An adaptive immune
response to a self antigen causing the observed pathology
is evidenced by the transfer of the disease by autoantigen
25 specific lymphocytes or antibodies. The pathogenic role of
the B cell response to the basement membrane antigen is
supported by the passive transfer of the disease to monkeys
with human autoantibodies eluted from the kidneys and by
the relative importance of the removal of the circulating
30 antibodies by plasma exchange in addition to the applica-
tion of immunosuppressive drugs in standard treatment
protocols.

The target of the toxic autoantibody response is the
non-collagenous domain of the $\alpha 3$ chain of type IV collagen
35 ($\alpha 3$ (IV)NC1), but not the homologous region of the $\alpha 1$ chain
($\alpha 1$ (IV)NC1). The epitope has been localized to $\alpha 3$ (IV)NC1

which is a major basement membrane component of glomeruli and alveoli; thus explaining the tissue distribution of the disease.

In 1984 the antigen was identified as a new subunit
5 of the NC1 domain of type IV collagen (Wieslander J. et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:3838). This resulted in the cloning of the antigen (Saus J. et al., 1988, J. Biol. Chem. 263:13374) and the designation as the $\alpha 3(\text{IV})$ chain. The epitope has been characterized as a
10 cryptotope, i.e. a denaturation of the antigen increases the reactivity with human sera. However, upon reduction the reactivity disappears (Wieslander J. et al., 1985, J. Biol. Chem. 260:8564), thus indicating a three dimensional epitope that normally is hidden inside the intact
15 glomerular basement membrane. The epitope has then been further studied and the reactivity of patient auto-antibodies has successfully been blocked with monoclonal antibodies (Pusey C. D. et al., 1987, Lab. Invest. 56:23; Hellmark T. et al., 1994, Kidney Int. 46:8263), indicating
20 a limited epitope recognition. One study has identified the C-terminal end of the $\alpha 3(\text{IV})\text{NC1}$ domain as a candidate epitope using synthetic peptides (Källuri R. et al., 1991, J. Biol. Chem. 266:24018). Others have pointed out the importance of the amino-terminal region of the NC1 domain
25 (Kalluri R. et al., 1996, J. Biol. Chem. 271:9062). The $\alpha 3(\text{IV})$ procollagen precursor sequence has been determined and is accessible from the Swiss-Prot database under AC:Q01955

A variety of studies have been performed on
30 experimental animal models of autoimmune diseases with the aim to identify antibody epitopes of potential pathogenic relevance. It has been shown (Hellmark T. et al., 1998, Kidney Int. 55:936) that the structural requirements essential for recognition by all patient sera might be
35 localized in the amino-terminal portion of the $\alpha 3(\text{IV})\text{NC1}$

domain. However, some sera also reacted with the central portion of $\alpha 3(\text{IV})\text{NC1}$.

Little is known about epitopes in human autoimmune diseases at a molecular level. This is most likely due to the methodological problems related to a diverse reactivity pattern of the autoantibodies. For example, the cysteine residues are critically involved in the folding of the NC1 domain of type IV collagen (Siebold B. et al., 1988, Eur. J. Biochem. 176:617). The reactivity of antibodies to the antigen is usually abolished if disulfide bonds are disrupted. This has for many years prevented the localization of the epitope within the NC1 domain by enzymatic digestion and such attempts have resulted in the loss of antigenicity.

Furthermore, the conformational requirements of the epitopes limit the application of linear synthetic peptides for the mapping strategies. In such studies one is limited to the use of areas of conformational flexibility such as the N-terminal and C-terminal regions because these regions of the native protein are assumed to exhibit the same spectrum of conformations as the synthetic peptide. A difficulty with this approach, however, is that due to the multiplicity of possibilities, antibody production against a desired conformation may represent a small portion of the antibodies raised and the process is relatively inefficient.

It is often of a great diagnostic benefit to determine quickly and safely the presence in body fluids of autoantibodies indicating Goodpasture's syndrome. Since the prognosis often is bad and often implies death of the patient a ready and effective diagnostic method is needed for determining whether these autoantibodies are present.

The object of the invention is to establish the target structure of the toxic antibody response in Goodpasture's syndrome, i.e. the conformational epitope

recognized by circulating autoantibodies from Goodpasture patients. On the basis of this target structure a method for a safe and accurate diagnosis, prognosis as well as therapy of Goodpasture's syndrome can be performed.

5 In order to achieve this purpose the method according to the invention has been given the characterizing features of claim 1.

In order to further explain the invention reference is given to the accompanying drawings in which

10 FIG 1 schematically illustrates the cloning strategy used to construct recombinant chimeric molecules of the amino-terminal domain of $\alpha 3(\text{IV})\text{NC1}$;

FIG 2 shows immunoblotting with serum from one patient (panel A) and anti-type X collagen monoclonal
15 antibodies (panel B) against four of the different chimeric proteins;

FIG 3 shows the immunoreactivity of a chimeric construct with sera from Goodpasture patients and healthy controls;

20 FIG 4 shows inhibition ELISA with recombinant chimeras blocking the reaction of patient sera to native purified $\alpha 3(\text{IV})\text{NC1}$; and

FIG 5 schematically illustrates the $\alpha 3(\text{IV})\text{NC1}$ domain with disulphide bonds and the 9 amino acid residues identified as the Goodpasture epitope.
25

In order to circumvent the above-mentioned problems an experimental procedure was chosen which allows for the expression of the antigen as a recombinant protein in a human cell line. This strategy enables the construction of
30 a variety of properly folded chimeric molecules, in which the $\alpha 3(\text{IV})\text{NC1}$ sequence harboring the Goodpasture epitope can be replaced to a varying extent by the corresponding sequence from the homologous $\alpha 1(\text{IV})\text{NC1}$ that is not recognized by the toxic autoantibodies in Goodpasture's
35 syndrome.

Since the toxic autoantibody response is highly selective for the $\alpha 3$ -chain of the heterotrimeric type IV collagen its immunoreactive domains were subjected to a mutation analysis by the replacements with corresponding sequences from the non-reactive highly homologous $\alpha 1$ chain in chimeric recombinant constructs. The high homology between both chains as well as conserved disulfide bonds enhanced the likelihood for the proper folding of the hybrid constructs as prerequisite for conformation dependent autoantibody binding.

The application of this strategy for the mapping of the Goodpasture epitope revealed nine amino acid residues in the amino terminal part of the $\alpha 3$ (IV)NC1 as the target structure recognized by all sera of 20 well defined patients with biopsy proven disease and thoroughly documented clinical outcome. Introduction of these nine amino acid residues from the $\alpha 3$ (IV) chain of NC1 into the wild type $\alpha 1$ (IV) sequence by replacement mutations converted the nonreactive $\alpha 1$ (IV)NC1 into a recombinant hybrid molecule that was recognized by all patient sera with the same affinity as the native purified Goodpasture antigen as shown by competition ELISA.

The identified nine amino acid residues are localized in the amino-terminal part of the $\alpha 3$ (IV)NC1 and form a discontinuous epitope in close vicinity to the cysteine residues that are critically involved in the folding of the NC1 domain (Siebold B. et al., 1988, Eur. J. Biochem. 176:617). The formation of disulphide bonds between the cysteine residues stabilizes the tertiary structure in the native NC1 domain and is expected to bring the identified amino acid residues more closely together. Conversely, it is very likely that the break of the disulfide bonding will affect the position of the critical residues dramatically which is in good agreement with earlier experimental

results that demonstrated the loss of immunoreactivity upon reduction of the Goodpasture antigen.

The close correlation between the specificity of the autoantibody response and clinical outcome indicates the presence of a conformational B-cell epitope which is recognized by circulating autoantibodies from apparently all Goodpasture patients. For the identification of the conformation dependent clearly pathogenic and critical epitope dominant negative mutations were introduced into the recombinant chimeric Goodpasture antigen by replacement of wild type $\alpha 3(\text{IV})\text{NC1}$ sequences by corresponding amino acid residues of the homologous non-reactive $\alpha 1(\text{IV})\text{NC1}$. Finally, the substitution of wild type $\alpha 1(\text{IV})\text{NC1}$ in nine discontinuous positions with amino acid residues from the $\alpha 3(\text{IV})\text{NC1}$ sequence resulted in a recombinant construct that was recognized by sera from all affected patients but in none of the healthy controls. The amino acid replacements define the target structure of the toxic antibody response in Goodpasture syndrome.

According to the invention the immunogenic region of the non-collagenous domain of the $\alpha 3$ chain of type IV collagen with intact cysteine residues is localized in the amino terminal end of the $\alpha 3$ chain, and at least five essential amino acid residues are exposed to circulating autoantibodies from Goodpasture patients. Four additional amino acid residues make up a conformational epitope of not more than nine amino acid residues, which is recognized by all autoantibodies from apparently Goodpasture patients. These nine amino acid residues correspond to amino acid no 1455, 1456, 1457, 1459, 1462, 1465, 1466, 1469, and 1495, respectively, in the $\alpha 3(\text{IV})$ procollagen precursor sequence. The amino acids in this amino terminal end of $\alpha 3(\text{IV})\text{NC1}$ have been identified as L-threonine, L-alanine, L-isoleucine, L-serine, L-glutamic acid, L-valine, L-proline, L-serine, and L-glutamine, respectively.

The immunogenic region according to the invention can be obtained by chemical or enzymatical cleavage of the native non-collagenous domain of the $\alpha 3$ chain of type IV collagen.

5 Recombinant chimeric molecules of the amino-terminal domain of $\alpha 3$ (IV)NC1 have been constructed. The proper antigen is expressed in a suitable human cell line and subsequently block the reaction of patient sera to native purified $\alpha 3$ (IV)NC1. Recombinant molecules can then be used
10 in a wide variety of applications.

The invention also refers to synthetic or recombinant polypeptides having as an antigenic region an amino acid sequence comprising between five and nine amino acid residues exposed to circulating autoantibodies from
15 Goodpasture patients. Polypeptides according to the invention are sufficiently short to be synthesized by means of chemical methods now standard in the art. Such polypeptides comprise no more than about 50-60 amino acid residues, several or all of the amino acids L-threonine, L-alanine, L-isoleucine, L-serine, L-glutamic acid, L-valine,
20 L-proline, L-serine, and L-glutamine in the polypeptide being capable of forming an epitope which immunoreacts with these autoantibodies. Further polypeptides are also contemplated as being within the scope of the present
25 invention, which polypeptides comprise the same epitope as a polypeptide having an amino acid sequence not significantly different than that represented by the amino-terminal domain of the $\alpha 3$ (IV) sequence shown in FIG 5. Such differences can easily be determined by, for example,
30 competitive immunoassay.

Recombinant or synthetic molecules harboring the immunogenic region according to the invention can then be used in a number of therapy forms in relation to Goodpasture's syndrome. For example, autoantibodies directed
35 against the basement membrane collagen type IV can be

extracorporeally eliminated by means of immunoadsorption. The epitope is immobilized by means of known techniques to a column with an inert material and a biological liquid, preferably blood plasma, is pumped through a system
5 comprising the affinity column.

An antigen composition comprising the immunogenic region according to the invention, which specifically reacts with autoantibodies present in body fluids from Goodpasture patients, can be used for blocking the
10 antibodies in the blood. The toxic autoantibodies can also be blocked by means of direct injecting to Goodpasture patients the epitope in the form of recombinant or synthetic molecules, an autoantibody-antigen complex being formed *in vivo* between the autoantibodies and the epitope
15 instead of between the autoantibodies and the glomerular basement membrane. The patient is treated in this way for two weeks while waiting for diminution of the immune response by the immune complex being removed in the liver.

Autoantibody producing cells can also be killed by
20 targeting these cells with an epitope-toxin complex. In this form of therapy a toxic substance to the cells is coupled to the recombinant or synthetic molecules according to the invention. The epitope binds to the cells and thus selectively affects them by releasing the toxic substance
25 to the cells.

The invention also refers to methods for the *in vitro* determination in body fluids of circulating autoantibodies from Goodpasture patients. The conditions are such that they permit an antigen antibody reaction which can be
30 demonstrated by means of standard physical or chemical means.

In such a method the inventive immunogenic region is brought into contact with a body fluid from a Goodpasture patient, which comprises circulating autoantibodies
35 directed against the basement membrane collagen type IV.

Preferably, a solid phase system is used. The solid phase can be a plastic surface, beads for aggregation or counting, paper, nitrocellulose filters etc.

In this connection the immunogenic region is coupled
5 to the inert carrier material in such a way that the immunogenic properties are not interfered with. The immunogenic region is preferably an integral part of the above-mentioned recombinant or synthetic molecules.

In the test systems synthetic or recombinant
10 polypeptides as well as enzymatically or chemically cleaved products of the native protein containing the immunogenic region according to the invention are coupled to the solid phase. Alternatively, these small immunogenic molecules are captured by monoclonal or polyclonal antibodies directed
15 against and reacting with the immunogenic region according to the invention, which in turn are coupled to the solid phase. A monoclonal antibody against the labelled immunogenic region can also be used in an inhibition assay.

Anti-idiotypic antibodies raised against the above-
20 mentioned antibodies are likewise coupled to solid phase for capturing circulating autoantibodies from Goodpasture patients. Anti-idiotypic antibodies are thus used for detecting patient antibodies or for assaying the epitope in a sample by means of competitive assay or double sandwich
25 assay. In this second method anti-idiotypic antibodies raised against poly- or monoclonal antibodies directed against the inventive immunogenic region are brought into contact with a body fluid from a Goodpasture patient, which comprises circulating autoantibodies directed against the
30 basement membrane collagen type IV.

After the addition of patient serum the system is washed. The conditions are such that they permit an antigen antibody reaction which can be demonstrated by means of standard physical or chemical means.

The detection system can be enzymatic, fluoresces, radioactivity, beads aggregated with a secondary antibody, all specific details in the test systems being well known for the skilled man within the art.

5 Poly- or monoclonal antibodies directed against and reacting with the immunogenic region according to the invention can readily be produced by a man skilled in the art. Likewise, anti-idiotypic antibodies raised against these antibodies should also be within the field of
10 expertise for a man skilled in the art. Such anti-idiotypic antibodies can for example be used for preventing an immunoglobulin from combining with the basement membrane antigen.

It is also believed that it is within the qualifications of a man skilled in the art to design a diagnostic
15 kit by means of previously known standard components and poly- or monoclonal antibodies directed against the immunogenic region according to the invention. A kit comprising recombinant or synthetic molecules with the
20 immunogenic region according to the invention should likewise lie within his normal field of expertise.

EXAMPLES.

For the construction of recombinant chimeric
25 molecules that harbor the Goodpasture epitope the amino terminal two thirds of the $\alpha 1$ chain of type X collagen including the leader sequence were fused to the NC1 domain of type IV collagen. The cloning strategy used to construct recombinant chimeric molecules is shown in FIG 1 under a.
30 (For experimental details see below and Table 2). In FIG 1 b denotes a sequence comparison of the $\alpha 3$ (IV) and $\alpha 1$ (IV) chains in the amino terminal part of the NC1 domain. Capital letters indicate borders of the constructs in the regional mapping. Amino acid residues are defined by means
35 of the single letter code. Numbers indicate the positions

(P1-P14) selected for replacement mutations. (For selection criteria see below).

The epitope mapping strategy with these constructs comprises four subsets of experiments: first the NC1 domain was divided into three parts and replaced the $\alpha 3(\text{IV})$ sequence by the corresponding $\alpha 1(\text{IV})$ sequence for each part separately; second the immunoreactive sequence was further narrowed down in the amino-terminal part of the $\alpha 3(\text{IV})\text{NC1}$ domain; third point mutations were introduced in the $\alpha 3(\text{IV})\text{NC1}$ in order to identify replacements with dominant negative effects on antibody recognition; and finally amino acid substitutions were performed in the $\alpha 1(\text{IV})\text{NC1}$ at selected positions that reconstitute the Goodpasture epitope in a domain that is normally not recognized by the patient sera.

Therefore further mapping studies remained confined to the amino-terminal part of the $\alpha 3(\text{IV})\text{NC1}$ using chimeric constructs in which the central and carboxyterminal parts of the NC1 domain were expressed as $\alpha 1(\text{IV})$.

Patients and sera.

Twenty samples with biopsy proven anti-GBM nephritis from the serum bank at the Department of Nephrology, Lund University were chosen and further analyzed in this study. All the patients had crescentic glomerulonephritis with linear deposits of IgG in direct immunofluorescence. Seven of the patients had in addition overt lung hemorrhage. Sera from 10 healthy blood donors were used as controls.

Antibodies.

The antibodies used were monoclonal antibodies against type X collagen (clone X53) (Girkontaite I. et al., 1996, Matrix Biol. 15:231), and the Mab17 monoclonal antibody raised against the $\alpha 3(\text{IV})\text{NC1}$ (Johansson C. et al., 1991, Connect Tissue Res. 25:229).

Cell culturing and transfection.

HEK-293 cells were cultured in 90 mm cell culture plates (Nunc, Roskilde, Denmark) in a DMEM:F12 1:1 medium with 5 percent fetal calf serum (Gibco-BRL, Paisley, GB).

5 For each construct 5 µg of the linearized plasmid DNA was transfected into 1×10^6 HEK-293 cells using an electroporator (BioRad, Hercules, CA), with the electrical settings 200V, 640µF in a 0.4 cm cuvette. The transfected cells were seeded on a new plate and after 48 hours

10 selection was started by supplementation with 800 µg/ml G-418 (Gibco-BRL, Paisley, GB). The medium was renewed every two days; the collection of supernatants was started when the G-418 resistant cells reached confluence. During the harvesting, the transfected cells were kept in FCS-free

15 DMEM/F12 supplemented with ascorbate (Schulte S. et al., 1998, J. Biol. Chem. 273:1551).

DNA constructs.

All restriction enzymes as well as ligase were

20 purchased from Boehringer-Mannheim, Mannheim, Germany. The primers used are shown in Table 1 below, and the Pfu-DNA polymerase was purchased from Stratagene.

Table 1. Primers used.

25	Primer no	Sequence 5' to 3'
	1	CATGCTGGATCCGGGGCCCCAGGCACCA
	2	GCTGGTGTGACAGCCAGTACGAGTACTCATT
	3	CTGGCTGTCAACCCCTGAGCCCATGCCCATGTC
	4	TGACATTCTAGAAATGGCGCACTTCTAAACTCC
30	5	CCATTCTAGAGTGTACGCGCGTGGGACCTGC
	6	CCATTGCGGCGCTTATGTTCTTCTCATACAGAC
	7	CCAGGGCCTCCTGGCTTTGGG
	8	GGCATTCAGGATTCCTGGG
	9	TCATGTCCAGAGGGGACAGTC
35	10	TGTCCCTCTGGACATGATGGGTCTATTGTTTGAC
	11	CCACAGTGTCTTCTGGGACC
	12	CCCAGAAGGACACTGTGGAATTGCTGTGGTTTGACTGTG
	13	ACAGTGCCACTCTACAGTGGGTACTCTTTGCTCTACG
	14	ACTGTAGAGTGGCACTGTCC
40	15	GGCAGCTGCCTGCAGCGATTTCAGCACAAATGCCCTTCC
	16	TCGCTGCAGGCAGCTGCC
	17	CCAGGCACCCCATCTGTTACGAGAGGCTTTGTCTTC

(Table 1. cont.)

18 AACAGATGGGGTGCCTGG
 19 ACCAGGCATAGTCAAACAACAGCAATTCCTTCATGTCC
 20 TGTTTGACTATGCCTGGTC
 5 21 GATCCACTGCTTCCACGTTT
 22 GTTGTCAGGTTCCAGGTGATCC
 23 GAAGACAAAGCCGTGCGTTGTCCA
 24 GTGTCGGGTGACGACAAAGCC
 25 GAAGGAATATCTGTGGTTTGACp5
 10 26 GGACATGAAGGATCTGCTGTGG
 27 CCCTCTGGACATTGAGGAATTGC
 28 GGCACGTGCCCCGATGGACATG
 29 CCACTGTAGAGTATCACTGTCCC
 30 GAAAACCCATGGTAGAGTGGC
 15 31 TTCCTTGTACATAAAGAAAAGA
 32 AGGCAGCTGCCAGCAGTTCCAAG
 33 GGTAATCGCGCAGGCAGCT
 34 GGCATTGTGCTAAATCGCTGC
 35 ACAATAGATATCCCATCGTGTCTCTGGG
 20 36 CCCAGAAGGACAGGATGGGATATCTATTGTTTG
 37 CTGGGACCAAACCTCTTTACAGCGGGTACTCTTTGC
 38 GCAAAGAGTACCCGCTGTAAAGAGGTTGGTCCC
 39 AGCTGCCGTGCAAAGTTCAGC
 40 GCTGAACTTTTCAGGCAGCTGC
 25

Construction of primary vectors.

The primary type X/type IV collagen chimera was constructed using the $\alpha 3(\text{IV})\text{NC1}$ cDNA cloned in pBluescript SK-vector (Stratagene, LaJolla, CA) with a BamHI site in
 30 the 5' end and a NotI site in the 3' end, and the full length type X collagen cDNA in the pBluescript SK-vector cloned between the HindIII and NotI sites. Both plasmids were cleaved with BamHI and NotI and the $\alpha 3(\text{IV})\text{NC1}$ cDNA was
 35 cDNA. This construct contains five unique restriction enzyme sites; a HindIII site in the 5' end of the construct, a BamHI site in the junction between the type X collagen and the type IV collagen NC1, HindII and XbaI in the $\alpha 3(\text{IV})\text{NC1}$, and a NotI site 3' of the coding DNA.

Dividing of the NC1 domain in three parts.

The BamHI, HindII, XbaI and NotI cleavage sites have been introduced into the $\alpha 1(\text{IV})\text{NC1}$ cDNA by PCR (Perkin-Elmer GeneAmp 2400, Foster City, CA), using primers 1-6.

Upon restriction with the appropriate enzymes the fragments of the $\alpha 1(\text{IV})\text{NC1}$ were used to replace the corresponding cassettes of the wild type $\alpha 3(\text{IV})$ sequence as previously described (Hellmark et al., 1998, Kidney Int. 55:936).

- 5 Since the major epitope region could be localized to the amino-terminal domain of the $\alpha 3(\text{IV})\text{NC1}$ this region was subjected to further mutational analysis. For the construction of six $\alpha 1/\alpha 3(\text{IV})$ chimeric NC1 domains the overlap extension PCR technique (Ho S. N. et al., 1989, Gene 77:51) was applied using the primers 6-20 in Table 1.

Replacement mutations of single amino acid residues in the $\alpha 3(\text{IV})$ sequence.

- Fourteen positions were selected for mutational analysis in a chimeric construct that harbors the amino-terminal $\alpha 3(\text{IV})\text{NC1}$ (from A to G in Figure 1). The selection criteria were based on identity in human and bovine $\alpha 3(\text{IV})$ sequences and concurrent non-conserved exchanges in the corresponding amino acid residues of the human $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ sequences. These 14 amino acid residues are emphasized in FIG 1 by the numbering of their positions in the sequence (1-14). The point mutations were introduced by the megaprimer method (Barik., S., 1993, In Methods in Molecular Biology, Vol. 15, B. A. White editor, Humana Press Inc., Totowa, New Jersey, 277-286) using the primers 6, 7, and 21-34.

Replacement mutations of single amino acid residues in the $\alpha 1(\text{IV})$ sequence.

- Finally, replacement mutations were introduced into chimeric constructs comprising the entire $\alpha 1(\text{IV})\text{NC1}$ domain. By site directed mutagenesis 5 and 9 codons were changed from wild type $\alpha 1(\text{IV})$ to the corresponding $\alpha 3(\text{IV})$, respectively. The substitutions were introduced by an overlap extension PCR (Ho S. N. et al., 1989, Gene 77:51)

using primers 6, 7 with 35-40 and primers 6, 7 with 15, 16, 19, 20, 39 and 40 respectively.

General handling of the constructs.

5 After sequencing, the constructs were restricted with HindIII and NotI and subcloned into a CMV promoter driven expression vector (pcDNA3, Leek, The Netherlands). All constructs were tested for translation of a protein with correct molecular weight using an *in vitro* system (Promega, 10 Madison, WI), with a S^{35} labeled cysteine and T7RNA polymerase. Before transfection, the plasmid DNA was linearized using ScaI.

Enzyme Linked Immunosorbent Assay (ELISA).

15 Native type IV collagen NC1 domains were purified as described elsewhere (Hellmark T. et al., 1994, Kidney Int. 46:8263), and coated at 0.5 μ g/ml. The coating efficiency of the different recombinant proteins was calibrated by their equal immunoreactivity with the anti-collagen type X 20 monoclonal antibody. Human sera were diluted 1/100 and the monoclonal antibodies 1/1000. The ELISA was performed following standard procedures (Hellmark T. et al., 1994, Kidney Int. 46:8263) using alkaline-phosphatase conjugated swine anti-human IgG (Orion Diagnostica AB, Trosa, Sweden) 25 as secondary antibody. For a positive ELISA, the absorbency value had to be higher than the mean value for the control sera plus two standard deviations. None of the control sera were positive in any ELISA.

30 **Inhibition ELISA.**

Dilutions of human sera were adjusted to give the same absorbency after one hour in a conventional ELISA with purified native type IV collagen NC1 domains. They were preincubated overnight at 4°C with different inhibitors; 35 i.e. recombinant proteins or purified native proteins in concentrations from 0.0025 to 25 μ g/ml. The amount of IgG that reacts with the surface bound Goodpasture antigen

despite the presence of inhibitor molecules in the fluid phase was determined after extensive washing procedures with alkaline-phosphatase-conjugated secondary antibodies as described above.

SDS-PAGE and immunoblotting.

One ml of the cell supernatants was precipitated with Triton X100 and trichloro acetic acid. The precipitate was applied to SDS-PAGE in 10-16 % gradient gels (SDS-PAGE) under nonreducing conditions (Laemmli U. K., 1970, Nature 227:680).

Immunoblotting experiments were performed on samples that were separated with SDS-PAGE and transferred to ImmobilonTMPVDF membrane (Millipore, Saint-Quentin, France) (Burnette W. N., 1981, Anal. Biochem. 112:195).

Example 1. Replacement of subdomains of the amino-terminal portion of the $\alpha 3$ (IV)NC1 by the corresponding $\alpha 1$ (IV) sequences.

In order to further narrow down the sequence that harbors the major epitope, six different constructs were generated that contain replacements of different parts of the amino-terminal domain of $\alpha 3$ (IV)NC1 by the corresponding sequences from the $\alpha 1$ (IV). These six chimeric proteins were designated D2 to D7 as shown in Table 2 below.

Table 2. Construction design (see FIG 1 for nomenclature and amino acid positions).

Name	Construct	Number of immunoreactive sera *
<i>Regional Mapping (Division of the NC1 domain)</i>		
D1	$\alpha 3$ from A to G then $\alpha 1$	20/20
D2	$\alpha 1$ from A to B then $\alpha 3$ to G then $\alpha 1$	20/20
D3	$\alpha 1$ from A to C then $\alpha 3$ to G then $\alpha 1$	20/20

(Table 2. cont.)

D4	$\alpha 1$ from A to D then $\alpha 3$ to G then $\alpha 1$	0/20
D5	$\alpha 3$ from A to E then $\alpha 1$	20/20
D6	$\alpha 3$ from A to F then $\alpha 1$	4/20
D7	$\alpha 3$ from A to D then $\alpha 1$	0/20

Replacement Mutations aimed at destroying the epitope
(Substitutions in the $\alpha 3$ (IV)NC1)

P1	$\alpha 3$ from A to G then $\alpha 1$; Substitution in position 1, D to S	20/20
P2	$\alpha 3$ from A to G then $\alpha 2$; Substitution in position 1, A to G	20/20
P3	$\alpha 3$ from A to G then $\alpha 3$; Substitution in position 1, R to H	20/20
P4	$\alpha 3$ from A to G then $\alpha 4$; Substitution in position 1, F to V	20/20
P5	$\alpha 3$ from A to G then $\alpha 5$; Substitution in position 1, A to D	20/20
P6	$\alpha 3$ from A to G then $\alpha 6$; Substitution in position 1, I to D	7/20
P7	$\alpha 3$ from A to G then $\alpha 7$; Substitution in position 1, S to Q	5/20
P8	$\alpha 3$ from A to G then $\alpha 8$; Substitution in position 1, E to S	20/20
P9	$\alpha 3$ from A to G then $\alpha 9$; Substitution in position 1, P to I	0/20
P10	$\alpha 3$ from A to G then $\alpha 10$; Substitution in position 1, S to H	0/20
P11	$\alpha 3$ from A to G then $\alpha 11$; Substitution in position 1, F to Y	20/20
P12	$\alpha 3$ from A to G then $\alpha 12$; Substitution in position 1, L to A	20/20

(Table 2. cont.)

P13	$\alpha 3$ from A to G then $\alpha 13$; Substitution in position 1, Q to R	4/20
P14	$\alpha 3$ from A to G then $\alpha 14$; Substitution in position 1, T to S	20/20
<i>Reconstruction of the epitope substitutions in the $\alpha 1$ (IV)NC1</i>		
S1	$\alpha 1$ from A; Substitutions in position 6 (D to I), 7 (Q to S), 9 (I to P), 10 (H to S), 13 (R to Q)	0/20
S2	$\alpha 1$ from A; Substitution in position 5 (D to A), 6 (D to I), 7 (Q to S), 8 (S to E), 9 (I to P), 10 (H to S), 13 (R to Q) and in addition I to T a.a.35, K to V a.a.45	20/20

* n = 20 patients with biopsy-proven Goodpasture disease. The immunoreactivity was assessed by ELISA experiments (see above for details).

It was found that all sera (n=20) reacted with the D2, D3, and D5 chimeric proteins while no antibody binding was detectable to D4 and D7. A subset of 4 sera also reacted with the D6 chimeric protein. The reactivity of the Goodpasture sera with the constructs indicates the localization of the major epitope to a region comprising the 42 amino acid residues between the positions C and F in FIG 1. However, for 20 percent of the sera the requirements for antibody recognition remained confined to the 15 amino acid residues of $\alpha 3$ (IV) from C to E in FIG 1.

Example 2. Amino acid substitutions in the $\alpha 3$ (IV)NC1 with dominant negative effect on autoantibody recognition.

Comparison of amino acid sequences for the $\alpha 1$ (VI) and $\alpha 3$ (IV) chain revealed 33 non-conserved residues in the

amino-terminal portion of the NC1 domain from position A to G in FIG 1. For the identification of those amino acid differences that might be of critical importance for antibody binding the sequence comparison was extended to

5 bovine $\alpha 3$ (IV) and human $\alpha 5$ (IV) sequences as bovine $\alpha 3$ (IV) is recognized by autoantibodies from Goodpasture patients in contrast to the non-reactive human $\alpha 5$ (IV). Only positions with identity in the human and bovine $\alpha 3$ (IV) sequences but with differences to the corresponding $\alpha 1$ (IV)

10 and $\alpha 5$ (IV) sequences were selected for the introduction of replacement mutations. This criterion was fulfilled by 14 amino acid residues, and their positions are indicated by the numbering of the positions (1-14) in FIG 1. In these positions the $\alpha 3$ (IV)-specific codons were replaced by the

15 corresponding $\alpha 1$ (IV) residues in the construct D1 (Table 2), resulting in different chimeric molecules that harbor a single amino acid exchange, named P1 - P14 in Table 2. Small differences in reactivity with the recombinant

20 proteins were obtained with sera from different individuals. However, two of the constructs, i.e. P9 and P13, at least partially abolished their reactivity. The results concerning the localization of critical positions for immunoreactivity by single amino acid residue

25 substitutions are in concordance with the effect of replacement mutations of longer stretches of the $\alpha 3$ (IV) sequence by the corresponding $\alpha 1$ (IV) domains in the aminoterminal portion of NC1. Thus, all positions that

30 turned out to be of essential importance localize within the same 42 amino acid residue long region (between C and F in FIG 1) that has been shown in the above described experiments to require $\alpha 3$ (IV) sequences for binding of the Goodpasture sera. In contrast to the majority of

35 Goodpasture patient samples (n=16) the reactivity of the four sera, which remained insensitive to the Q to R mutation in the P13 construct, had earlier been shown to be

restricted to a subdomain of the critical region (from C to E in FIG 1) which does not include position P13.

Example 3. Construction of the Goodpasture epitope by site-directed mutagenesis in the $\alpha 1(\text{IV})\text{NC1}$.

5 Based on the knowledge of positions in the $\alpha 3(\text{IV})\text{NC1}$, in which single replacement mutation can destroy the immunoreactivity, the Goodpasture epitope was reconstructed within the frame of the non-reactive $\alpha 1(\text{IV})\text{NC1}$ by site-directed mutagenesis of five and nine amino acid residues respectively. First, the five critical residues in position 10 6, 7, 9, 10, and 13 with the dominant negative effect on autoantibody binding were replaced in the $\alpha 1(\text{IV})\text{NC1}$ by the corresponding amino acid residues, I, S, P, S, and Q from 15 the $\alpha 3(\text{IV})$ sequence (construct S1 in Table 2). These five amino acid residues are essential for the immunoreactivity of the Goodpasture epitope as will be shown below.

 Second, four additional non-conserved residues localized in the region between C and E in FIG 1 were 20 substituted. The resulting $\alpha 1(\text{IV})\text{NC1}$ construct harbors all eight amino acid substitutions from the $\alpha 3(\text{IV})$ sequence between C and E in FIG 1 and the amino acid exchange R to Q in position P13 (construct S2 in Table 2). Likewise, as 25 will be shown below, these four amino acid residues are important for the most efficient target structure of the antibody response in Goodpasture's syndrome but they are not essential for the immunoreactivity of the Goodpasture epitope.

 FIG 2 shows immunoblotting of four different chimeric 30 proteins with serum of a representative Goodpasture patient (panel A) and with a control monoclonal antibody (mAb; murine anti-collagen X) (panel B). Lane 1: recombinant $\alpha 3(\text{IV})\text{NC1}$; lane 2: D1 construct; lane 3: S1 construct; lane 4: S2 construct. The upper row (arrowhead) indicates 35 bands in which specific reactivity has occurred, thus indicating that the control antibody recognized all four

constructs. In contrast, the patient antibodies bound to $\alpha 3(\text{IV})\text{NC1}$, D1 and S2, but not to the S1 construct containing the five amino acid substitutions which were shown to be insufficient to reconstitute ELISA-reactivity in the frame of $\alpha 3(\text{IV})\text{NC1}$ (cf. Table 2 above). Bands of low molecular weight in panel A are likely due to non-specific reactivity to minor contaminants from the cell culture medium, whereas in panel B (lane 1) the bands may indicate partial degradation of the $\alpha 3(\text{IV})\text{NC1}$.

The first construct, S1, gave only a weak reactivity with Goodpasture sera in immunoblotting experiments and the mutational changes were insufficient to make the molecule reactive with patient sera in ELISA. The second chimeric protein, S2, was on the other hand recognized by all sera in both immunoblotting (FIG 2) and ELISA; thereby indicating that all reactivity of the Goodpasture autoantibodies is directed towards this limited region in the NC1 domain of collagen type IV.

FIG 3 shows the immunoreactivity against the chimeric construct S2 (see Table 2 above for details) of Goodpasture sera ($n=20$), 13 patients without overt lung hemorrhage (filled circles) and 7 patients with lung hemorrhage (open circles), as well as age-matched controls ($n=10$) (open squares). ELISA experiments showed a clear distinction between the Goodpasture patients and the healthy control populations. The cut-off value (mean value of the healthy controls + 2 SD) is indicated by a horizontal line.

As shown in FIG 3 there was no difference between epitope specificities of autoantibodies from patients with or without lung bleeding when the chimeric construct S2 was used. In contrast to the 20 Goodpasture patients, there was no reactivity in the 10 sera from healthy humans used as control.

Example 4. Inhibition ELISA.

For the evaluation of the affinity of Goodpasture autoantibodies for the different chimeric proteins inhibition ELISAs have been performed using recombinant $\alpha 3(\text{IV})\text{NC1}$, D1, S1, and S2 to block the binding to native NC1 domains from type IV collagen. The data confirm that the major epitope in Goodpasture's syndrome is preserved in the S2 chimeric protein.

The affinity of Goodpasture antibodies to the chimeric constructs is shown in FIG 4. Here all the inhibition ELISA curves were derived from one representative patient. The antibody binding to the native $\alpha 3(\text{IV})\text{NC1}$ coupled to a solid phase was inhibited by the soluble recombinant $\alpha 3(\text{IV})\text{NC1}$, D1, and S2 chimeric proteins, but not by the S1 construct. For $\alpha 3(\text{IV})\text{NC1}$, D1, and S2 the decline in immunoreactivity indicates a comparable affinity below saturation levels. At saturation the inhibition capacity of D1 and S2 remained at 85 % level of that of $\alpha 3(\text{IV})\text{NC1}$, thus indicating that only 15 % of the auto-antibodies recognize structures not contained in the immunodominant 9 amino acid residues but which are fully contained in the S2 construct (cf. Table 2).

As seen in FIG 4, the reaction could be inhibited to 85 % with both the D1 and S2 chimeric proteins, and that the affinity is approximately the same. The recombinant $\alpha 3(\text{IV})$ could inhibit the reaction to 100 %, hence 15 % of the autoantibodies are directed against other areas of the $\alpha 3(\text{IV})$ chain than the N-terminal of the $\alpha 3(\text{IV})\text{NC1}$ in this specific serum. This tendency was shown to be true for all sera, i.e. the D1 and S2 inhibited the antibody binding less than the recombinant $\alpha 3(\text{IV})\text{NC1}$, nonetheless all sera had the majority of their autoantibodies directed against the epitope expressed on the S2 construct.

FIG 5 is a schematic drawing of the secondary structure of the $\alpha 3(\text{IV})\text{NC1}$ domain involved in Goodpasture

autoimmunity. The immunogenic region of this domain is based on the results obtained with the chimeric construct S2 and includes two crossing disulfide bonds (indicated by solid lines between cysteine residues with roman numerals).
5 Each amino acid of the linear sequence is represented by circles. The filled circles emphasize the critical nine amino acid residues identified, and they are all clustered in the close vicinity of the disulphide bonds of the cysteine residues I-IV. It should be noted that these bonds
10 can exist between the cysteine residues I-II and II-IV as well as between the cysteine residues I-IV and II-III.

As shown in FIG 5 the identified critical nine amino acid residues are all localized in the amino terminal part of $\alpha 3(\text{IV})\text{NC1}$ and form a discontinuous epitope in close
15 vicinity of the those cysteine residues which are involved in the folding of the NC1 domain. The formation of disulphide bonds between cysteine residues stabilizes the tertiary structure of the native NC1 domain, the critical amino acid residues being brought into a spatial rela-
20 tionship which is important for antibody recognition. Conversely, it is very likely that breakage of these disulphide bonds would dramatically affect the position of the critical residues, a result which would be consistent with earlier results documenting loss of immunoreactivity
25 upon reduction of the Goodpasture antigen.

CLAIMS

1. Immunogenic region comprising a part of the non-collagenous domain of the $\alpha 3$ chain of type IV collagen
5 having intact cysteine residues, said part being recognized by circulating autoantibodies from Goodpasture patients, characterized in that said part comprises at least five amino acid residues which are substantially non-adjacent in the amino acid sequence, is located in the
10 amino terminal end of said $\alpha 3$ chain, and is exposed to said autoantibodies.

2. Immunogenic region as claimed in claim 1, characterized in that said part comprises not more than nine amino acid residues.

15 3. Immunogenic region as claimed in claim 2, characterized in that said not more than nine amino acid residues corresponds to amino acid no 1455, 1456, 1457, 1459, 1462, 1465, 1466, 1469, and 1495, respectively, in the $\alpha 3$ (IV) procollagen precursor sequence.

20 4. Immunogenic region as claimed in claim 3, characterized in that said amino acids comprise L-threonine, L-alanine, L-isoleucine, L-serine, L-glutamic acid, L-valine, L-proline, L-serine, and L-glutamine, respectively.

25 5. Polypeptide molecules comprising an immunogenic region as claimed in any of claims 1 to 4.

6. Polypeptide molecules comprising an immunogenic region which is not significantly different than that claimed in any of claims 1 to 4 as determined by competitive immunoassay.

30 7. Recombinant molecules comprising the immunogenic region as in any of claim 1 to 6.

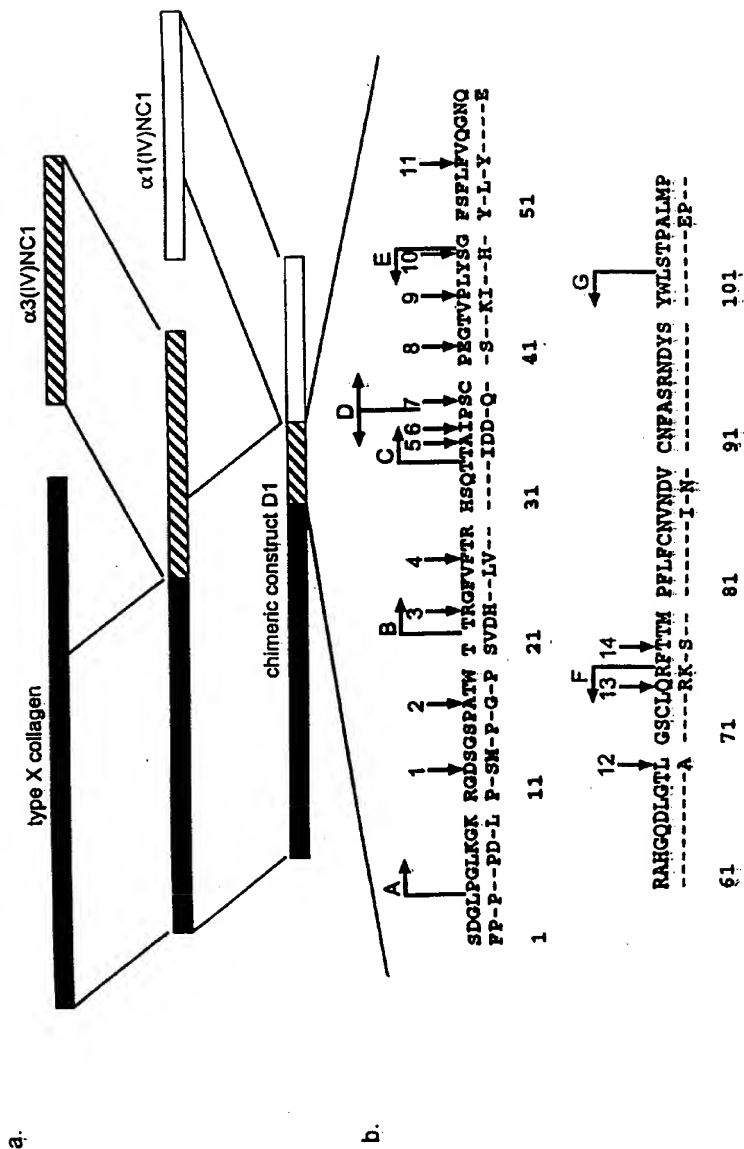
8. Synthetic molecules comprising the immunogenic region as in any of claim 1 to 6.

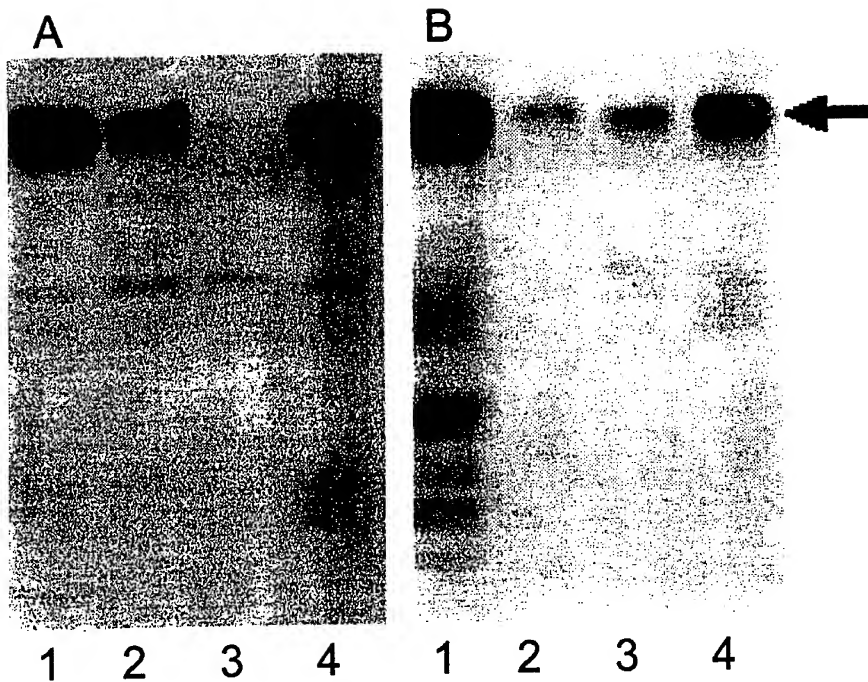
35 9. A diagnostic kit containing immunogenic molecules as in any of claim 5 to 8.

10. Polyclonal antibodies excluding autoantibodies from Goodpasture patients, which are directed against and reacting with the immunogenic region as in any of claim 1 to 4.
11. Monoclonal antibodies directed against and reacting with the immunogenic region as in any of claim 1 to 4.
12. Polyclonal antibodies directed against and reacting with the immunogenic molecules as in any of claim 5 to 8.
13. Monoclonal antibodies directed against and reacting with the immunogenic molecules as in any of claim 5 to 8.
14. A diagnostic kit containing poly- or monoclonal antibodies as in claim 10 to 13.
15. Anti-idiotypic antibodies raised against antibodies of claim 10 to 13.
16. Antigen composition comprising the immunogenic region as claimed in any of claims 1 to 8, which specifically reacts with autoantibodies present in body fluids from Goodpasture patients.
17. Method for the *in vitro* determination in a body fluid of circulating autoantibodies from Goodpasture patients, characterized in that the immunogenic molecules as claimed in any of claims 5 to 8 are brought into contact with said body fluid under conditions which permit an antigen antibody reaction, said reaction then being demonstrated by means of physical or chemical means.
18. Method as claimed in claim 17, characterized in that said immunogenic molecules are coupled to a carrier material.

19. Method for the determination in a body fluid of circulating autoantibodies from Goodpasture patients, characterized in that anti-idiotypic antibodies as claimed in claim 15 are brought into contact with said body fluid under conditions which permit an antigen antibody reaction, said reaction then being demonstrated by means of physical or chemical means.

Fig. 1





3/5

Fig. 3

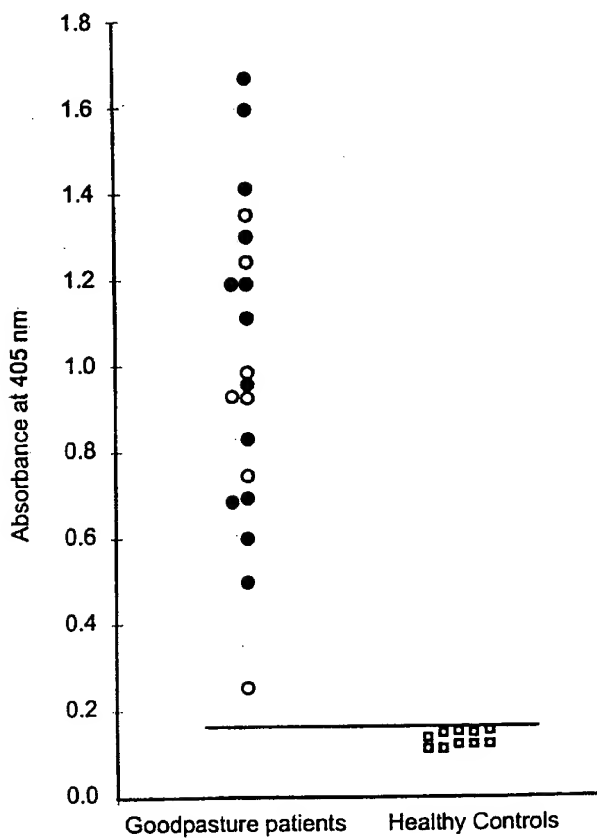
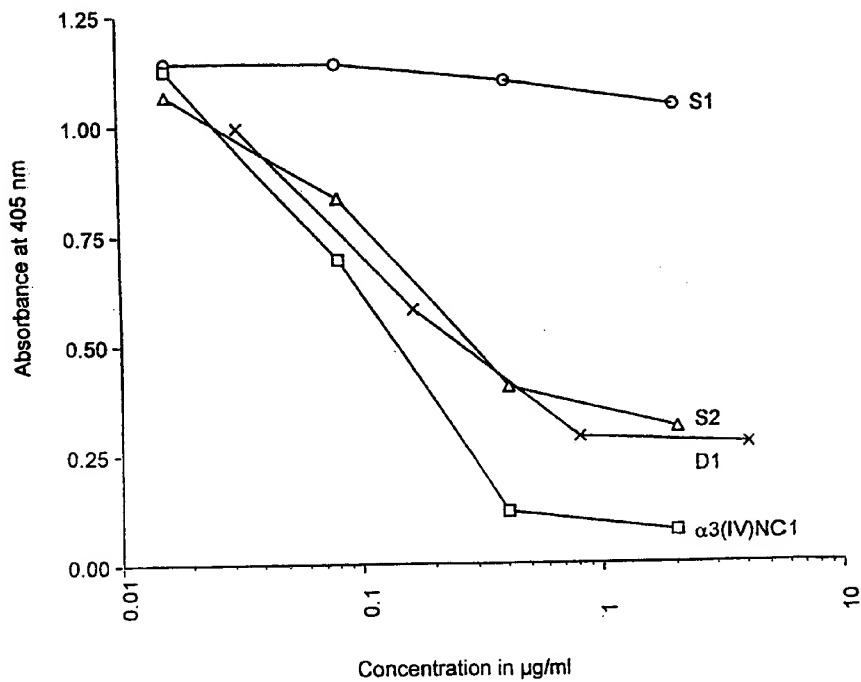


Fig. 4



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/01416

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 33/564, C07K 16/18, C07K 16/18, C07K 14/78
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Journal of Biological Chemistry, Volume 271, No 15, 1996, Raghu Kalluri et al, "The Goodpasture Autoantigen. Structural delineation of two immunologically privileged epitopes on alpha3(IV) chain of type IV collagen", page 9062 - page 9068, see the "discussion"	1,2,5-14, 16-19
	--	
X	Clin Exp Immunol, Volume 113, No 17, 1998, J. J. Ryan et al, "Recombinant alpha-chains of type IV collagen demonstrate that the amino terminal of the Goodpasture autoantigen is crucial for antibody recognition", page 17 - page 27, see fig. 8 and "Discussion"	1,2,5-14, 16-19
	--	

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

- * Special categories of cited documents
- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed
- * "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- * "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- * "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- * "&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

7 December 1999

12-01-2000

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/01416

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Kidney International, Volume 53, 1998, Kevin E.C. Meyers et al, "Human Goodpasture anti-alpha3(IV)NC1 autoantibodies share structural determinants. Rapid Communication" page 402 - page 407 --	1,10-13,14
A	Journal of the American Society of Nephrology, 1997, ThomasHellmark et al: "Comparison of Anti- GBM Antibodies in Sera With or Without ANCA", page 376 - pages 385 --	1
A	US 5424408 A (STEPHEN T. REEDERS ET AL), 13 June 1995 (13.06.95) -- -----	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

02/11/99

PCT/SE 99/01416

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5424408 A	13/06/95	NONE	

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Tumstatin sequence
 pink = residues in claim 4 of WO 00/11475

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 65 70 75 80
 Asn Val Asn Asp Val Cys Asn Phe Ala Ser Arg Asn Asp Tyr Ser Tyr
 85 90 95
 Trp Leu Ser Thr Pro Ala Leu Met Pro Met Asn Met Ala Pro Ile Thr
 100 105 110
 Gly Arg Ala Leu Glu Pro Tyr Ile Ser Arg Cys Thr Val Cys Glu Gly
 115 120 125
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